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14. ABSTRACT

Spider silks have the potential to provide new bio-based materials for numerous military applications ranging from protective clothing to parachute cords to composite materials in aircraft. Specific amino acid motifs have been identified which have been conserved for over 125 million years in all spiders using their silk to physically trap their prey. No one has systematically varied the sequence motifs in the spider silk proteins and determined how this influences the mechanical properties of the resulting fibers. These experiments will provide the predictive knowledge enabling the design of materials with very specific elastic and strength properties for each military application.

Specific Aims

1) The properties of dragline silk are the result of the combining both proteins MaSp 1 and 2.
2) The elasticity of the individual molecules and the materials will be proportional to the number of elastic motifs they contain and varying the amount of the non-elastic regions will vary the tensile strength.

15. SUBJECT TERMS
spider silk, protein, fiber, spinning

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Objectives: No changes

Status of Effort:

All the synthetic genes have been constructed and expressed. Several have been spun into fibers and mechanically testing done on those fibers. New purification methods have been developed for both the bacterially expressed proteins and the proteins produced in milk. Post spin draw methods were developed that greatly increased elasticity and/or strength. NMR provided the best structural description yet for the proteins in the fiber and also now allows us to determine the protein secondary structure on samples as small as 1 mg of synthetic spider silk.

Accomplishments:

YEAR 1.

Bacteria were genetically engineered to produce two spider silk protein variants composed of basic repeat units combining a flagelliform elastic motif ($[GPGG\mathbf{X}]_4$) and a major ampullate silk strength motif ([linker/poly-alanine]. The secondary structures of the pure recombinant proteins in solution were determined by circular dichroism. The data presented suggest that the nature of the 5^{th} and 10^{th} amino acid (**X**) in the $[GPGG\mathbf{X}]_2$ elastic motif and temperature have an impact on the amount of β-sheet structures present in the proteins. More specifically, increasing temperatures seem to be positively correlated with β-sheet formation for both proteins and this state is irreversible or reversible when both **X** (5^{th} and 10^{th}) in the elastic motif are hydrophilic or hydrophobic respectively. Moreover, each pure silk-like protein was able to spontaneously self-assemble into films from aqueous solutions. Two kinds of synthetic fibers were made by pulling fibers from these preassembled films as well as spinning fibers from each protein resolubilized in HFIP. The mechanical data show that the pulled fibers are far tougher than the spun fibers suggesting a better fiber organization.

Fiber Type	Total of Fibers	Diameters µm	Young's Modulus MPa	Maximum Stress MPa	Maximum Extension %	Toughness MJ/m^3
$A1S8_{20}$ P	15	12.20 ± 4.99	1706.8 ± 791.87	28.64 ± 8.41	18.99 ± 12.88	3.41 ± 2.61
$A1S8_{20}$ S	19	32.15 ±16.24	759.68 ± 540.27	28.58 ± 17.18	3.72 ± 1.24	0.464 ± 0.30
$Y1S8_{20}$ P	31	15.79 ± 6.05	1081.49 ± 1000	49.64 ± 19.35	34.06 ± 25.30	10.6 ± 10.2
$Y1S8_{20}$ S	18	28.4 ± 11.32	933.62 ± 727.14	10.21 ± 7.32	1.59 ± 1.03	0.089 ± 0.11

Table 1

Table 1: Mechanical testing data.
Average values measured for all types and kinds of synthetic fibers. The standard deviation of each value is indicated (± STD). P = pulled; S = spun.

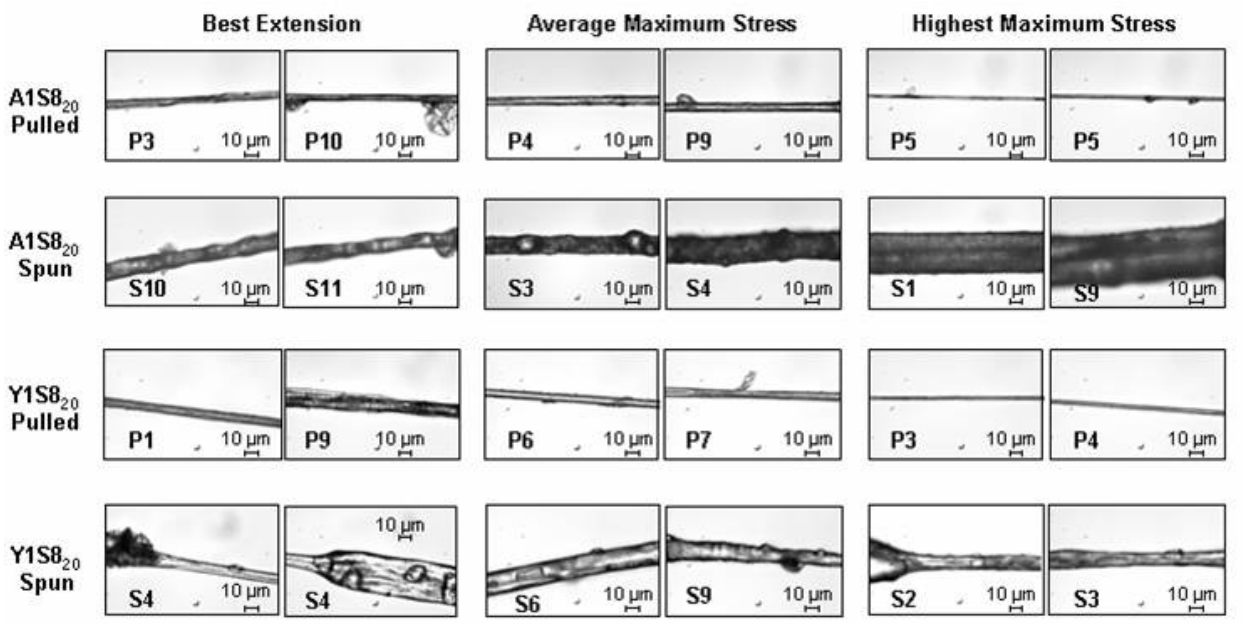


Fig. 1: Pictures of pulled and spun A1S8$_{20}$ and Y1S8$_{20}$ fibers.
For each fiber type, the pictures taken before tensile tests show the fibers that achieved the best extension, best maximum stress and average maximum stress. The fibers shown here correspond to the ones plotted in the stress/strain curves (Fig. 5): fiber type (P = pulled fiber; S = spun fiber) and identity (number) of each individual fiber is indicated in bold in the bottom left corner of each picture.

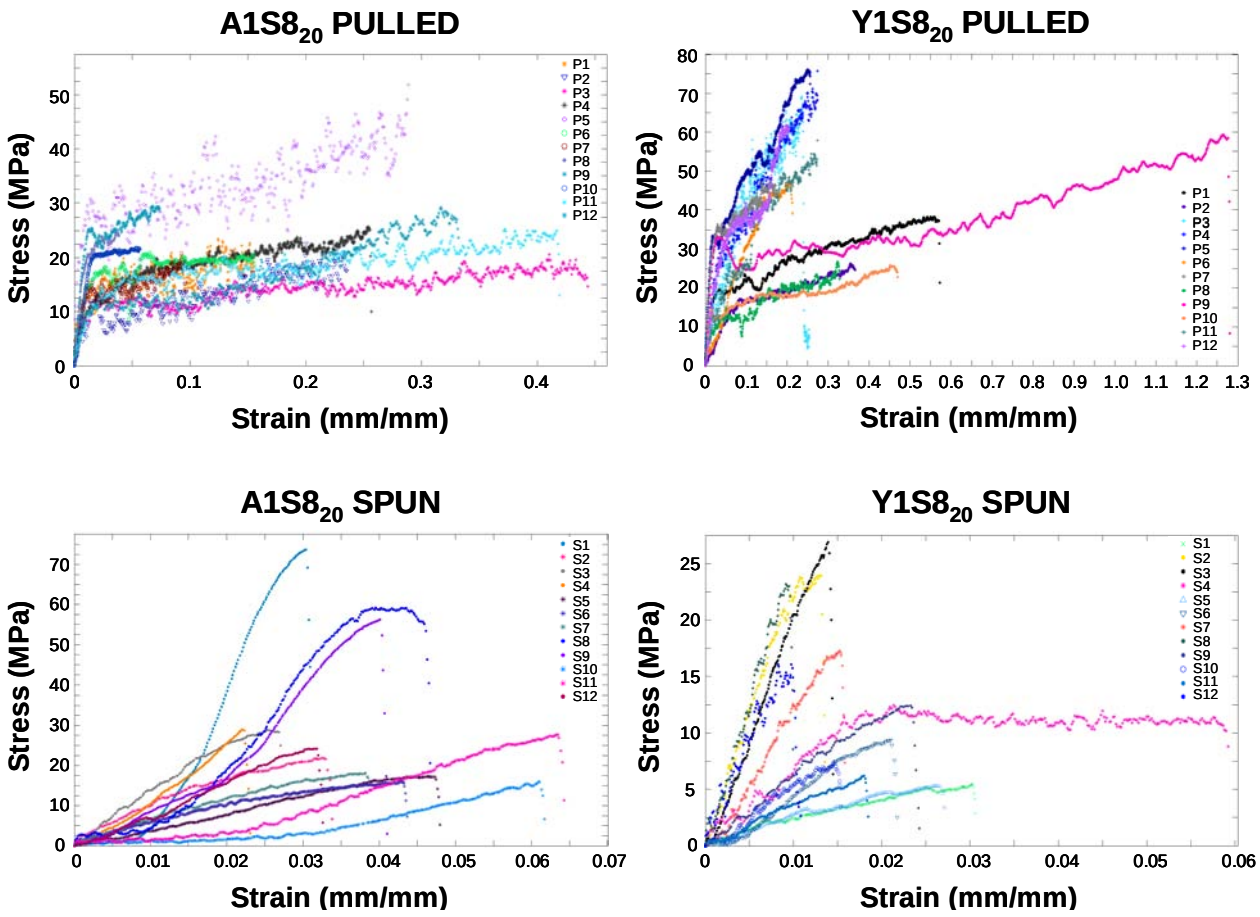


Fig. 5

Fig. 2: Stress/Strain curves of the synthetic fibers.
P1-P12= Pulled fibers 1 through 12; S1-S12= Spun fibers 1 through 12.
Note that the scales for both stress and strain differ from one graph to another.

We have developed a two-step process for purification of both MaSp1 and MaSp2 from goat's milk. As part of our continuing work with Nexia Biotechnologies we acquired most of their supply of goat's milk containing the two spider silk proteins. The milk, however, had been stored for over three years and we found their published purification methods were not successful. So we started over and found conditions that greatly increase solubility and also create a two-step process utilizing tangential flow and a column step using the AKTA system. We have not yet started spinning fibers from these proteins due to our efforts to optimize the purification methods as we have over 400 L of milk still stored containing approximately 1 kg of silk proteins.

In another development with Nexia we have purchased most of the founder goats they produced in order to protect the genetics they developed. Some of those goats have not been bred and others were hormonally induced to lactate. As of this date we have not received permission from the USDA to import them to the US from Canada. The regulations for import are currently being revised and we expect to receive permission when that is completed.

YEAR 2.

The first major advance concerns out NMR efforts to understand the basic protein structures in the fiber. To that end we have discovered methods to obtain huge enrichments in the natural fibers of both 13C and 15N amino acids (Fig. 3). We have gotten as high as 60% isotope enrichments. This may sound low but in looking at previous data from out lab and others, the highest possible reported to date has been 12-15%. In addition have not gotten labeling of both proline and tyrosine (interestingly we had to use phenylalanine to get tyrosine as tyr itself leads to no incorporate label), which has not been observed before. We have also been able to label other amino acids via long-term feeding due to metabolic transfer of label to other key amino acids. In a JACS paper we are able to confirm that the GGX sequence is not in a ß-sheet but is in a Gly II helix as we predicted several years ago. We are now finishing the data on the proline label that for the first time will confirm the presence of ß-turns for the GPGGX sequences and we hope to also confirm the spiral structure of ell.

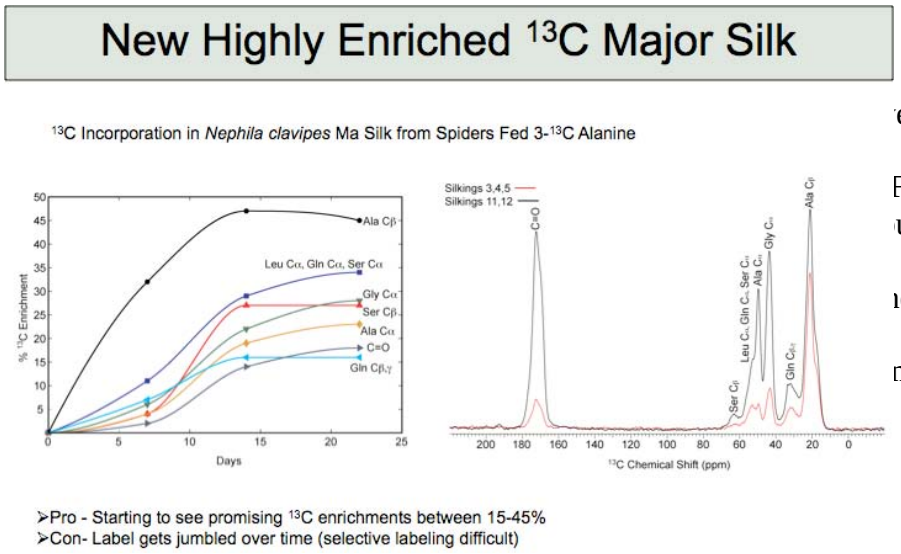


Fig. 3. 13-C labeling of natural major ullate silk with Ala. Spiders were fed the amino acid ıeir water for and the silk mined by NMR after varying lengths of time.

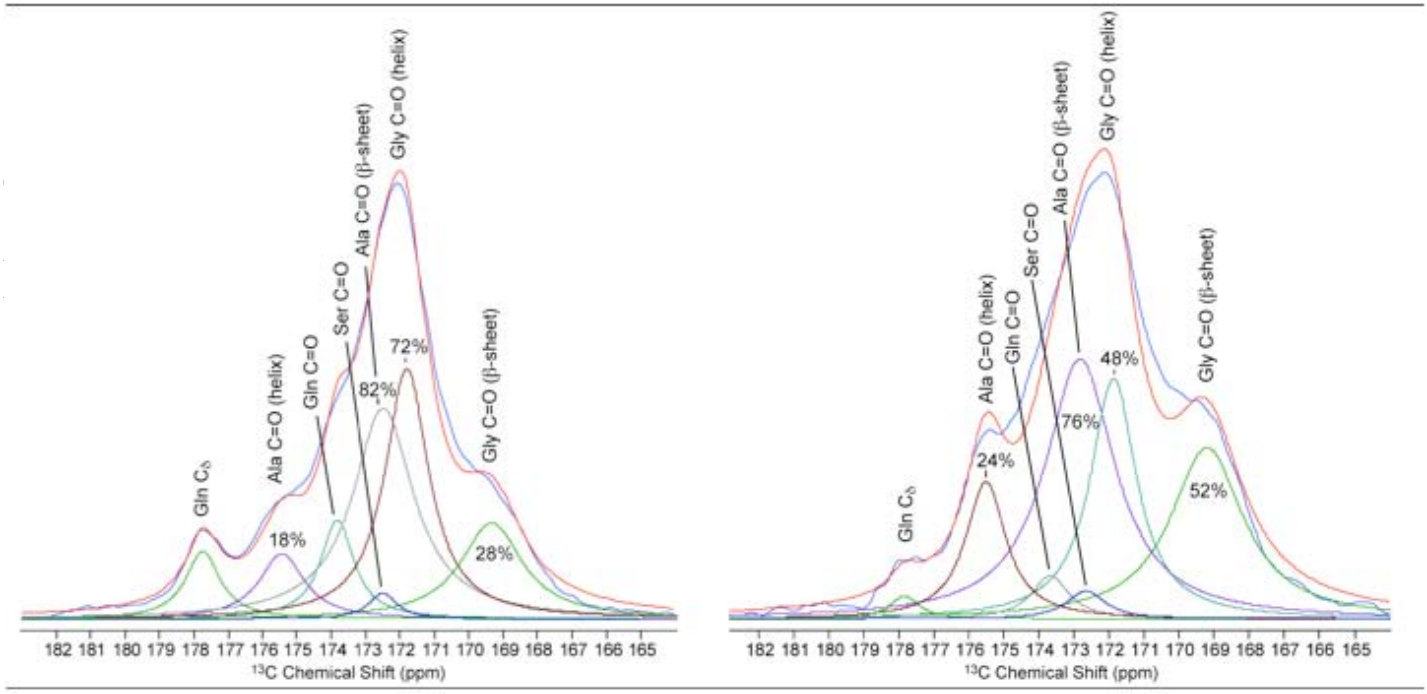


Figure 4 Carbonyl region from fully relaxed 13C DD-MAS NMR spectrum of water wetted (a) Ma silk and (b) Mi silk. The spectra were fit to extract the percent of Gly and Ala that each adopt both β-sheet and helical conformations.

The second major advance is the ability to conduct post-spin draw on fibers spun from organic solvents and then placed in water for the draw. This leads to nearly a 10-fold increase in tensile strength and up to 100-fold increases in elongation. The elongations are now at the same amount as natural fibers and the tensile strength is within a factor of three. We have also clearly delineated differing behaviors in spinning, post-spin draw behavior and mechanical properties between different protein sequences. In addition we have demonstrated that without the presence of a poly-alanine in the repeat the proteins will not form fibers. These were all done on GPGGX sequences based on the flagelliform silk.

The third is the expression purification and spinning of proteins differing in the length of the poly-ala segment from 4-16. All these were successfully spun. In fact the poly-ala 4 produced fibers in excess of 2 meters long. We were also able to directly apply a post-spin draw ratio of 2 for these fibers during the spinning process without the need for water. The mechanical testing is just now being done but the fibers are much thicker in diameter (60-70 um) than the flagelliform based fibers suggesting that we can use much higher post-spin draw ratios.

The final one is we completed and published (Brooks, et al) a comprehensive study of spinning solutions for MaSp 2 protein from goats. This clearly shows that both elongation and tensile strength can be substantially altered by the spinning solution. It also confirmed that the use of HFIP and isopropanol gives the best mechanical properties. We are now moving into a similar smaller study to determine the best spinning parameters for the combined MaSp 1 and 2 solutions.

We have further refined a two-step process for purification of both MaSp1 and MaSp2 from goat's milk. As reported last year we purchased most of the founder goats Nexia produced in order to protect the genetics they developed. After a 9 month odyssey of bureaucratic hassles we will brought the goats into othe US and have begun milk production and protein purification.

YEAR 3.

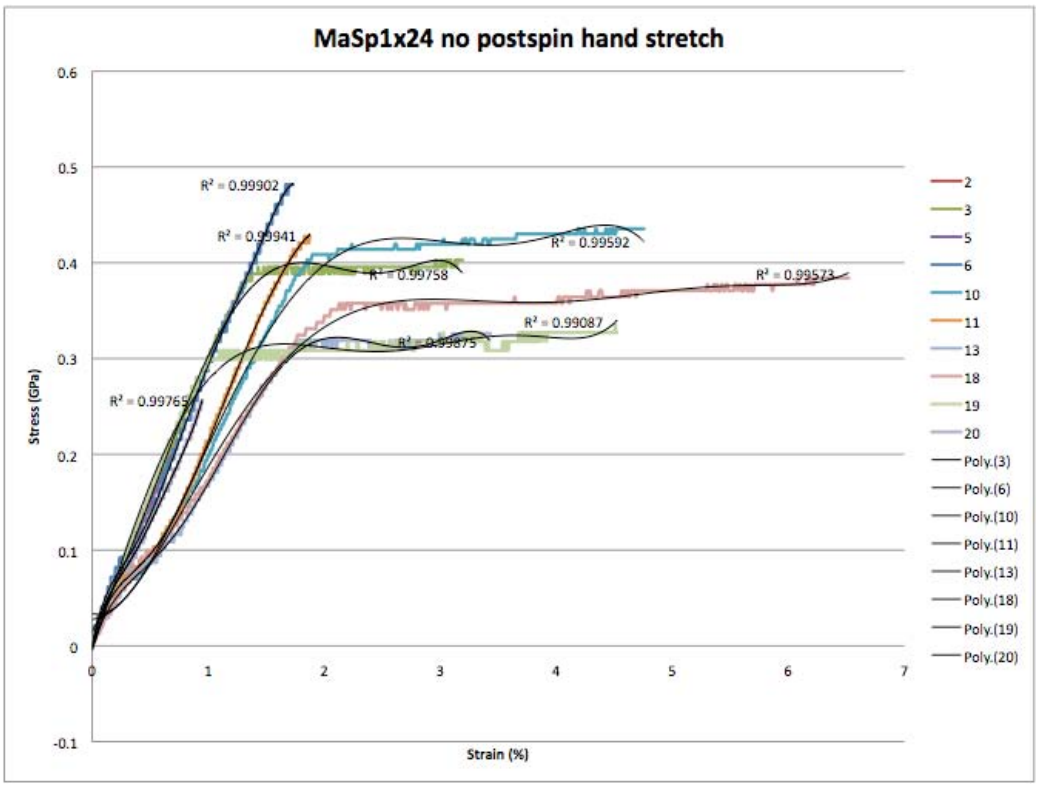
There have been a number of major advances this year. We have developed several methods for post-spin draw on fibers. A summary of the mechanical properties of one set of fibers is presented below (Table 2) as an example of the work we are currently doing. These fibers are based on flagelliform elastic sequences with major ampullate strength and linker sequences.

Parameters *vs.* Treatments		**Young's modulus (GPa)**	**Max. stress (MPa)**	**Max. strain (%)**	**Toughness (MJ/m^3)**
$A1S8_{20}$ fibers	As-spun	2.3	74.1	6.2	1.3
	As-pulled	3.6	56.2	44.4	9.28
$A1S8_{20}$ S fibers	1-step draw: IPA	1.4	32.4	27	6.9
	2-step draw: IPA/water	7.1	151.8	70	69.4
	1-step draw: MeOH	1.8	33.2	5.6	1.2
	2-step draw: MeOH/water	1.6	52.42	27.9	10.6
$Y1S8_{20}$ fibers	As-spun	2.7	27.4	5.9	0.6
	As-pulled	2.4	56.7	79.6	17.2
$Y1S8_{20}$ P fibers	1-step draw: water	8.3	143.1	80.3	61.6
Native silks	Dragline	11-13	1,100	30	160
	Flagelliform	0.003	500	270	150

Table 2. Mechanical properties of $A1S8_{20}$ and $Y1S8_{20}$ fibers after various treatments.

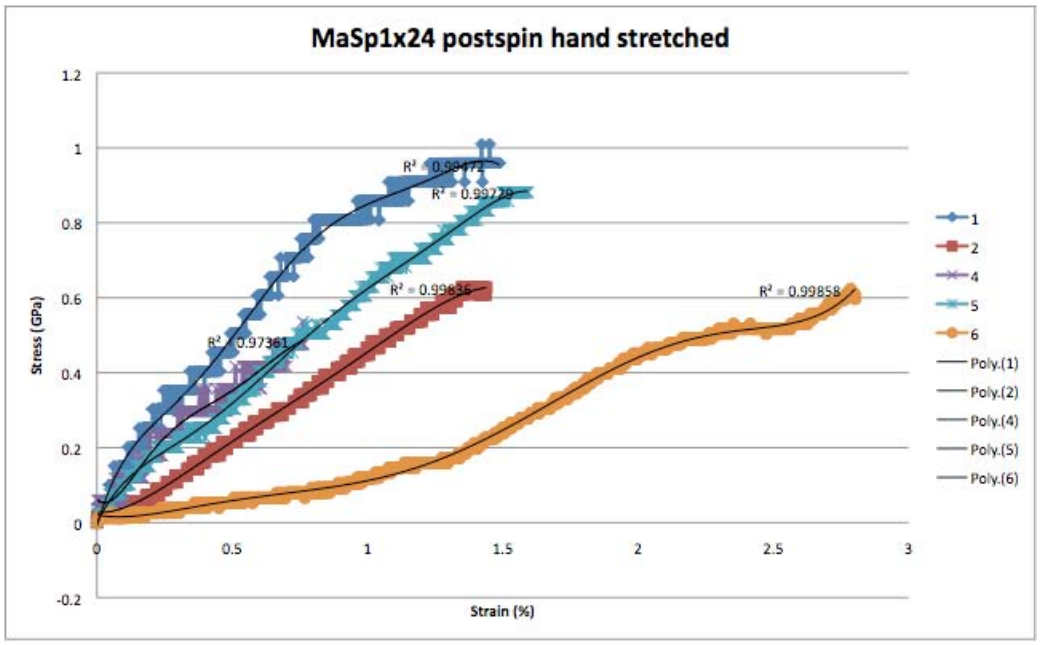
The best values obtained for each type of fiber for several mechanical parameters are indicated. The 'pulled' fibers (P) and 'spun' (S) fibers were generated in aqueous and organic conditions respectively.

For each post-spinning modifications of the $A1S8_{20}$ spun fibers, the fibers were subjected to single- or double-step post-spinning modifications (1-step or 2-step) including drawing (DR= 1.5 each time) after soaking in different solvent baths.



For the modified $Y1S8_{20}$ pulled fibers, the fibers were drawn (DR= 1.5) after soaking in water.

Since there is interest in defense materials with high strength and low elongation we have focused on the MaSp 2 protein, which is the most likely to provide those properties. The two figures below show the properties of various single fbers of that protein with and without post-spin draw. The improvement after draw is remarkable as is the lack of increased extension in some fibers.



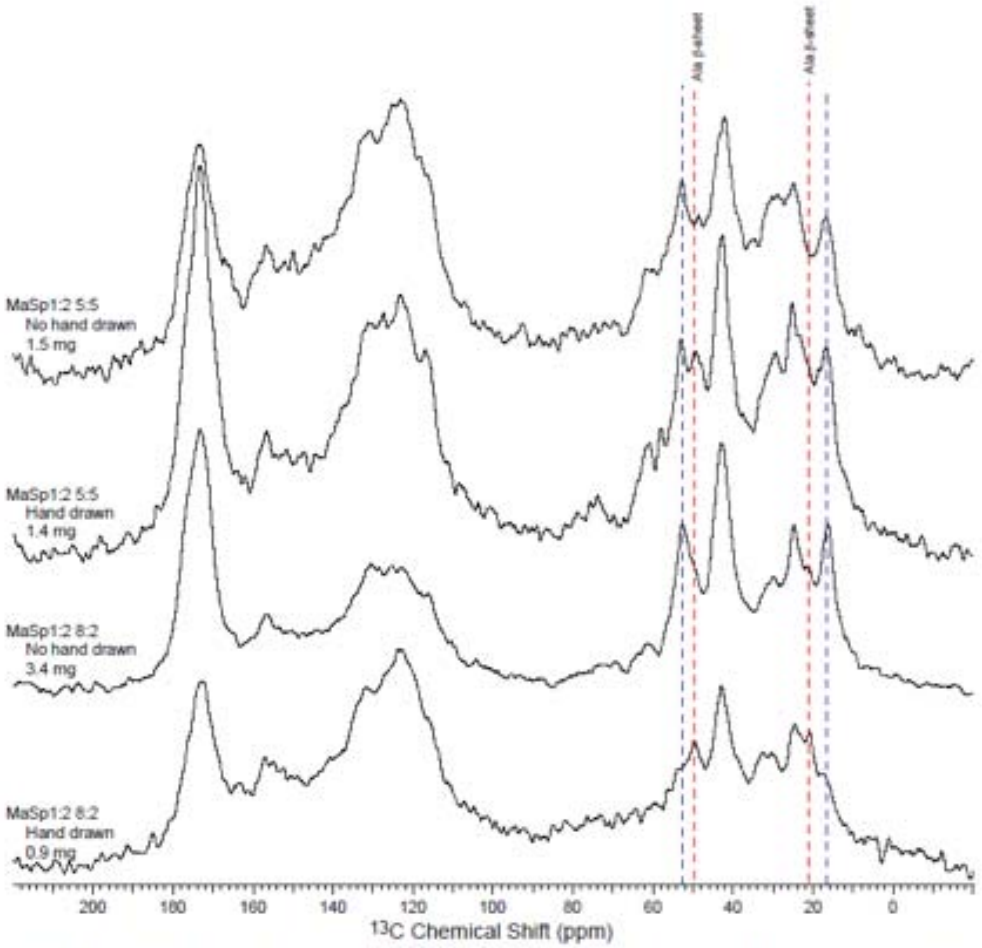


Figure. Bo's Samples - ^{13}C CP-MAS of synthetic fibers. CP was collected at 10kHz MAS, with 10k scans and 1k complex points collected. Exponential line broadening of 100 Hz was applied and the data was zero filled to 2k complex points. All data was collected on a Varian VNMRS 400 MHz wide-bore spectrometer with a 3.2 mm triple resonance probe operating in double resonance ($^{1}H/^{13}C$) mode.

We have also made substantial progress on NMR analysis of very small samples. We can now get decent specta on about 1 mg of fiber (Fig. 5). The data are good enough to correlate with other spectra to determine what changes have occurred in processing or between different protein fibers.

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Three manuscripts submitted and three in preparation.

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Interactions/Transitions: Discussions with Goodyear Tire and Nike are currently under way.

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